STUDIES ON COMPLEMENT FIXATION

IV. ON THE AFFINITY OF SHEEP CORPUSCLES FOR ANTISHEEP HEMOLYSIN *

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A knowledge of the factors which affect the affinity of sheep corpuscles for antisheep hemolysin is of importance in all complement-fixation studies in which a sheep cell system is employed. It gives a measure of the rapidity of sensitization of sheep corpuscles in the presence of hemolysin. It indicates also quantitative means for the removal of so-called natural hemolysin from serums employed in complement-fixation tests. It was indeed this latter phase which led us to undertake this investigation. We had become convinced that the presence of antisheep hemolysin in some human serums interferes with the correctness of complement-fixation tests. It will be recalled that this source of error was one of the important factors which led Noguchi to devise the "human system" as a means for the diagnosis of syphilis. In discussing the results of a number of parallel tests which gave sharper results with the Noguchi as compared with the Wassermann procedure, this investigator [1] states: "Just how this difference in sharpness of reaction between my system and that of Wassermann arises has been repeatedly emphasized and there can be no doubt that this is due to the occasional excessive antisheep amboceptor present in some human sera under investigation." On the other hand, it is well known that in laboratories, where an average of 100 or more tests are examined daily, the sheep cell system is considered more practical than the Noguchi system. The original purpose, therefore, of this investigation was to study the affinity of sheep cells for antisheep hemolysin quantitatively with a view of finding whether a simple procedure could be devised for removing natural hemolysin from human serum without unduly delaying the completion of large numbers of Wassermann tests. Fortunately, several preliminary measurements of the rate of absorption of antisheep hemolysin by sheep cells, carried

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*Preliminary Report, Abst. of Bacteriol., 1921, 5, p. 24.

[1] Serum Diagnosis of Syphilis, Ed. 2, p. 122.

out by one of us,[2] suggested a simple and desirable method for hemolysin removal. The quantitative studies on the affinity of sheep cells for hemolysin were continued, however, and this paper will consider the following three phases: (1) the rate of absorption of hemolysin by sheep cells at different temperatures; (2) the effect of the concentration of hemolysin on the absorption capacity of a given amount of sheep cells; (3) the time and temperature of sensitizing sheep cells in the presence of hemolysin.

EXPERIMENTS

The hemolysin employed was prepared by immunizing rabbits with concentrated sheep cells, previously washed 5 times with normal salt solution. The animals were given 3 intravenous injections of packed cells at 48-hour intervals. The first injection consisted of 1 c c and the second and third of 2 c c each. Five days after the last injection, a hemolysin titer of 1:2,000 was usually obtained.

Complement was obtained by bleeding guinea-pigs under anesthesia directly from the heart. The blood was placed in the icebox immediately after clotting, and the clear serum was drawn off in about 15 hours after bleeding. From 4 to 5 pigs were bled at a time in order to insure the uniformity of the complement. No complement was used unless it was free from nautral hemolysin and possessed a good hemolytic titer.

The sheep cells were obtained from our own sheep kept for the purpose of supplying blood to the Wassermann laboratory. The cells were washed 4 times with salt solution and after the fourth, or final, washing, they were packed by centrifugation for 14 minutes at 1,500 revolutions per minute employing the same length of time and speed in each case. The concentrated cell suspension was employed for hemolysin extraction, while a 5% saline suspension was used in the hemolytic phase of these experiments. The latter is frequently spoken of as the standard sheep cell suspension.

Hemolysin considered in this paper is, in practically every case, expressed in units, and a unit was taken to be the smallest quantity which hemolyzed 0.1 c c of the standard sheep cell suspension in the presence of 0.1 c c of pooled 1:10 guinea-pig complement after 15 minutes' incubation in the water bath. This unit was determined by preparing a series of dilutions of hemolysin serum and titrating each

[2] Kahn, R. L.: Jour. Lab. and Clin. Med., 1921, 6, p. 218

with 0.1 c c of the sheep cell suspension and 0.1 c c of pooled complement. These titrations were carried out in a series of 10 tubes, in the proportions shown in table 1.

The units were read after 15 minutes' incubation in the water bath, and the number of units contained in the undiluted hemolysin serum computed accordingly. Thus, if a serum dilution of 1:2,000 gave a titration unit of 0.05 c c, 1 c c of undiluted serum contained 40,000 units. (1 unit in 0.05 c c or 20 units in 1 c c; 20 × 2,000= 40,000.)

TABLE 1

PROPORTIONS IN WHICH TITRATIONS WERE CARRIED OUT IN TEN TUBES

Tubes	Hemolysin, C c	Complement (1:10) C c	Sheep Cells 5%, C c	Saline, Drops
1	0.1	0.1	0.1	3
2	0.09	0.1	0.1	3
3	0.08	0.1	0.1	3
4	0.07	0.1	0.1	3
5	0.06	0.1	0.1	4
6	0.05	0.1	0.1	4
7	0.04	0.1	0.1	4
8	0.03	0.1	0.1	5
9	0.02	0.1	0.1	5
10	0.01	0.1	0.1	5

1. THE RATE OF ABSORPTION OF HEMOLYSIN BY SHEEP CELLS AT DIFFERENT TEMPERATURES

To begin with, the rate of absorption of hemolysin by sheep cells at room temperature was studied. In the first absorption experiment, a dilution of hemolysin, each c c containing 20 units was employed. One c c quantities of this dilution were pipetted into 6 Wassermann tubes and 0.05 c c of packed sheep cells added to each. Extraction of hemolysin was permitted in the first tube for 5 minutes; in the second, for 10 minutes; in the third, for 15; in the fourth, for 20; in the fifth, for 25; and in the sixth tube for 30 minutes. Each tube was rapidly centrifuged at the end of its extraction period and the supernatant fluid drawn off.

Hemolysin titrations were carried out with the supernatant fluids of each of the 6 tubes in accordance with the following scheme:

Supernatant fluid (c c)	0.5	0.3	0.1
Complement (c c)	0.1	0.1	0.1
Sheep cells (c c)	0.1	0.1	0.1

The results after 15 minutes' incubation in the water bath showed no hemolysis in any of the tubes in the quantities of supernatant fluid employed, indicating that the hemolysin originally contained in the solutions (20 units) was absorbed in every case.

This experiment was then repeated employing 1 c c quantities of hemolysin solution containing 100 units of hemolysin, with the same results. In other words, 0.05 c c of packed sheep cells extracted 100 units of hemolysin in 5 minutes at room temperature.

In the next step, six 1 c c quantities of hemolysin solution, each containing 200 units of hemolysin, were employed. Into each of these 6 tubes were added 0.05 c c packed sheep cells and, as in the previous experiments, extraction permitted for 5, 10, 15, 20, 25 and 30 minute periods. Each tube was centrifuged after its extraction period and the supernatant fluid again titrated in the presence of complement and sheep cells for unextracted hemolysin. The titrations were carried out in the same manner as indicated in the first experiment. It was found that 0.5 c c quantities of supernatant fluid of the tubes which underwent 5 minutes' extraction showed slight hemolysis after 15 minutes' incubation in the water bath. Half c c quantities of the supernatant fluids of tubes which received longer extraction periods showed, in each case, a trace of hemolysis, only.

Our next problem was to find whether the same amount of packed cells added to 1 c c human serum containing 200 units of hemolysin would extract this quantity in this short period. This was of importance, in view of the fact that in complement-fixation tests the problem involved is the removal of natural hemolysin from serum. Accordingly, 0.01 c c of undiluted hemolysin containing 200 units was added to 1 c c quantities of pooled human serum and extractions carried out with 0.05 c c quantities of packed sheep cells at 5, 10, 15, 20, 25 and 30 minute intervals. The human serum was obtained by mixing serums sent to this laboratory for Wassermann tests, after completing the examinations. It was found that the supernatant fluids obtained on centrifugation after these extraction periods were free from hemolysin, indicating extraction of the 200 hemolysin units originally contained in these solutions.

Having shown that 0.05 c c of packed sheep cells were capable of absorbing close to 200 units of hemolysin from saline solutions and the same number from serum solutions after short extraction periods at room temperature, our next problem was to find to what extent

the temperature affects hemolysin extraction, and, also, whether the cells from different sheep show variations in the amount of hemolysin extracted. It was also decided to use 400 instead of 200 units of hemolysin. The extraction periods in these experiments were 5, 10, 15 and 20 minutes, and the temperatures were water bath (37.5 C) room (18-24 C.) and icebox (8-12 C.).

The protocol of the first experiment is herewith given in detail:

A hemolysin solution was prepared in the proportion of 0.01 c c undiluted hemolysin to 1 c c of salt solution. One c c of this solution contained 400 units, determined by a hemolysin titration according to the procedure indicated in the foregoing. One c c quantities of this hemolysin solution were added to a series of 12 tubes. The packed sheep cell suspension was then prepared by

TABLE 2

EXPERIMENT 1

Time of Adding Sheep Cells	Extraction Temperatures											
	Room (21 C.)				Water Bath (37.5 C.)				Icebox (10 C.)			
	Cell Suspension				Cell Suspension				Cell Suspension			
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 1	Tube 2	Tube 3	Tube 4	Tube 1	Tube 2	Tube 3	Tube 4
	C c	C c	C c	C c	C c	C c	C c	C c	C c	C c	C c	C c
9:00 a. m.	....	....	....	0.05	....	....	....	0.05	....	....	....	0.05
9:05 a. m.	....	....	0.05	....	....	....	0.05	....	....	....	0.05	....
9:10 a. m.	....	0.05	....	....	....	0.05	....	....	....	0.05	....	....
9:15 a. m.	0.05	....	....	....	0.05	....	....	....	0.05	....	....	....
9:20 a. m.	All tubes were centrifuged at high speed and supernatant fluid drawn off											

washing 4 times with salt solution in the usual manner and centrifuging for 14 minutes at 1,500 revolutions per minute; 0.05 c c quantities of this suspension were then added at different intervals to each of the 12 tubes. These intervals were so arranged as to give each series of 3 tubes an extraction period of either 5, 10, 15 or 20 minutes, in accordance with table 2. (Each tube contained 1 c c hemolysin solution containing 400 units.)

In order to find the number of hemolysin units extracted in each case, a regular hemolysin titration was carried out with each of the supernatant fluids. The readings of these hemolysin titrations, and the computed number of hemolysin units extracted by the sheep cells at different periods and temperatures is given in table 3.

The computation of the number of hemolysin units extracted at each of the temperatures was a relatively simple matter. Thus, in the case of supernatant fluid 1 (room temperature) and its unit of 0.03, the number of units extracted by the sheep cells was obtained in the

following manner: 1 unit of hemolysin is contained in 0.03 c c; 33.3 units of hemolysin are contained in 1 c c; originally 1 c c contained 400 units; 400 — 33.3 = 366.7.

The experiment just described was repeated in every detail with pooled human serum instead of salt solution. This was prepared by adding undiluted hemolysin to serum in the proportion of 0.01 c c to 1 c c of serum. Each c c in this case also contained 400 units of hemolysin. The results obtained with these serum solutions were similar to those obtained with the salt solutions, except that there were a somewhat larger number of units extracted from the serum compared with the salt solution.

TABLE 3

RESULTS OF HEMOLYSIN TITRATION

Tubes	Temperature of Extraction	Time of Extraction in Minutes	Titration Readings, C c	Number of Hemolysin Units Extracted
1	Room	5	0.03	367
	Water bath	5	0.02	350
	Icebox	5	0.02	350
2	Room	10	0.03	367
	Water bath	10	0.03	367
	Icebox	10	0.02	350
3	Room	15	0.03	367
	Water bath	15	0.03	367
	Icebox	15	0.02	350
4	Room	20	0.03	367
	Water bath	20	0.03	367
	Icebox	20	0.02	350

These hemolysin extraction experiments carried out in each case with salt and serum solutions were repeated 11 times, using the corpuscles of 5 different sheep. The cells of 1 sheep were employed in 3 experiments; the cells of each of the others, in 2 experiments.

In view of the fact that the number of hemolysin units extracted in each of these experiments closely approximate one another, the table representing the results of each of the 11 experiments is not given. Instead, the average findings of these experiments are presented.

The corpuscles from the 5 different sheep did not show any marked variation in their hemolysin absorption capacity. It will be noted that the extraction at water bath temperature was greater than at room temperature and this, in turn, greater than at icebox temperature. But the differences were comparatively small. Neither were there marked differences in hemolysin absorption between 5 and 20 minutes.

It might be added also that in some of these experiments, ordinary sized drops of packed cells were employed instead of 0.05 c c quantities. This was done because in the procedure for removing natural hemolysin, proposed by one of us,[2] the addition of drops of packed sheep cells per 1 c c quantities of serum is recommended. It was desired, therefore, to establish that practically the same results will be obtained with a drop as with 0.05 c c of packed cells.

2. THE EFFECT OF THE CONCENTRATION OF HEMOLYSIN ON THE ABSORPTION CAPACITY OF 0.05 C C OF PACKED SHEEP CELLS

There seems to exist much difference of opinion among investigators as to the amount of hemolysin a given quantity of cells is capable of absorbing in a given time. This becomes evident when

TABLE 4

RATE OF ABSORPTION OF ANTISHEEP HEMOLYSIN BY SHEEP CORPUSCLES AT DIFFERENT TEMPERATURES

Time of Extraction, Minutes	Number of Units of Hemolysin Extracted from a Solution of 400 Units at					
	Water Bath Temperature from		Room Temperature from		Icebox Temperature from	
	Salt Solution	Serum Solution	Salt Solution	Serum Solution	Salt Solution	Serum Solution
5	376*	399	363	380	356	361
10	382	399	377	388	370	371
15	390	400	384	392	376	377
20	388	400	387	395	379	379

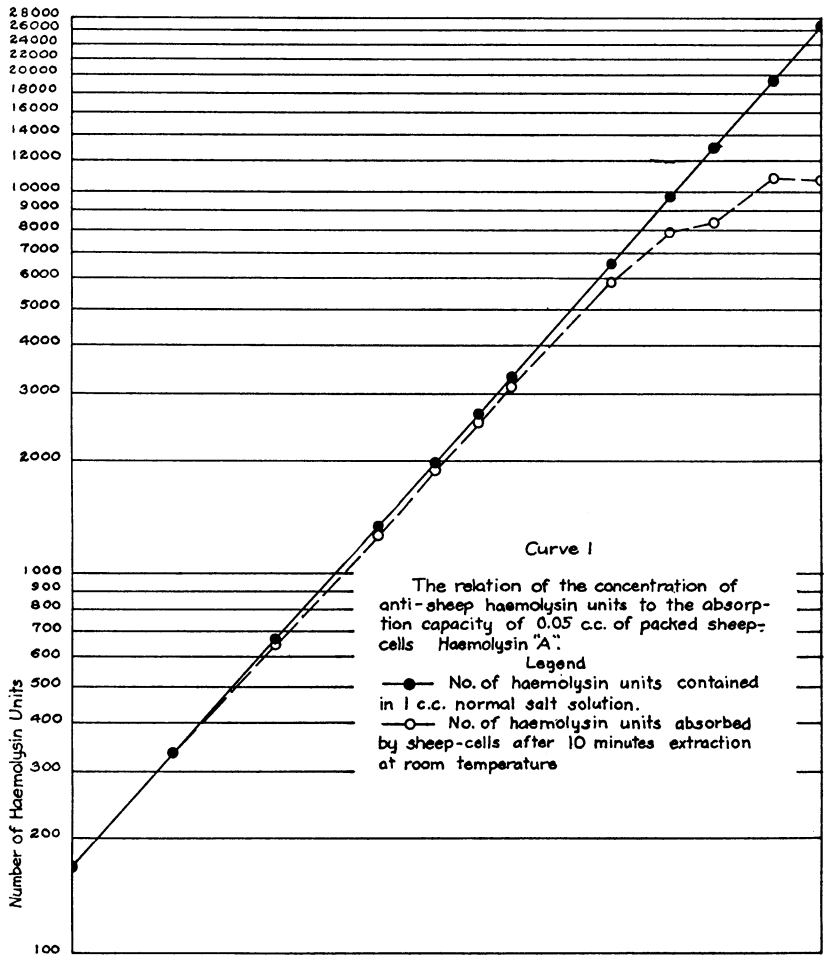
* Each number represents an average of 11 different extraction experiments.

one considers the time and temperature for sensitization of sheep cells, as recommended in three recently standardized Wassermann procedures. Thus Neil [3] of the Hygienic Laboratory recommends mixing the sheep cells with hemolysin and letting the mixture remain for 15 minutes at room temperature; Hinton,[4] for ½ hour at 37 C.; and Kolmer,[5] for 1 hour at room temperature. That sheep corpuscles are capable of absorbing huge quantities of specific hemolysin has been shown by Morgenroth and Arrhenius.[6] It seemed advisable, however, to attempt to find the absorption capacity of 0.05 c c of packed sheep cells when exposed to different concentrations of hemolysin for 10 minutes at room temperature. This quantity of sheep cells was chosen

[3] Public Health Reports, 1918, 33, p. 1387.
[4] Jour. of Syph., 1920, 4, p. 598.
[5] Ibid., p. 616.
[6] Quoted by Arrhenius, S.: Immunochemistry, p. 150.

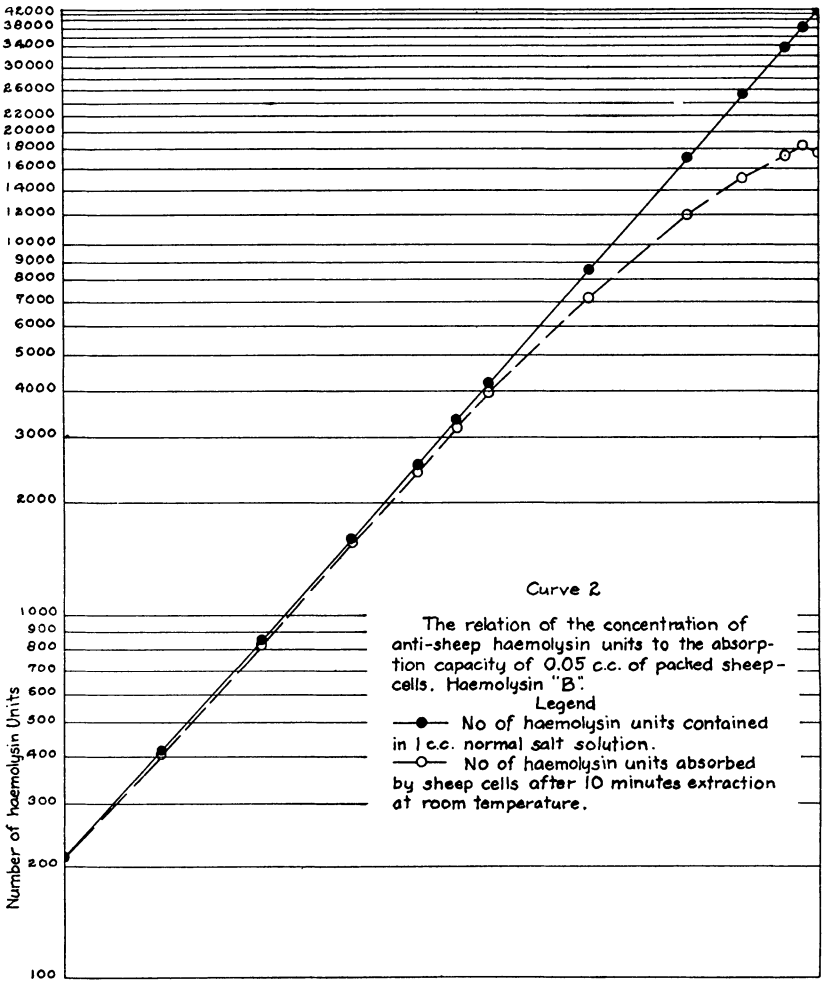
because it is equivalent to 1 c c of a 5% suspension—a quantity which represents the amount of cells employed in the regular Wassermann procedure.

Ten different stock solutions of antisheep hemolysin were used in these experiments. The general plan was to pipet various gradations



of hemolysin serum into a series of Wassermann tubes. These gradations ranged from 1 c c to 0.01 c c. To those which contained less than 1 c c, sufficient salt solution was added to make up to this quantity. To each tube was then added 0.05 c c of packed sheep cells, carefully mixed and allowed to remain for 10 minutes at room temperature. The

tubes were then centrifuged at high speed and the supernatant fluid drawn off. Regular hemolysin titrations were carried out before subjecting the serum to the sheep cells, and with the supernatant fluids after extraction, to determine in each case the number of hemolysin



units per c c and thus determine the number of units extracted by the corpuscles.

The results of two such experiments are given in table 5. To what extent the number of units of hemolysin absorbed depends on the concentration of hemolysin is particularly well illustrated in charts 1 and 2.

It will be noted that the number of hemolysin units are indicated on an arbitrary line and the number of absorbed units are diverging from this line little by little until 10,000 or more units are reached. The main reason why the absorption lines do not follow the original lines to the end is because of the marked agglutinating power of undiluted (or little diluted) hemolysin serum on sheep cells. Thus, when 0.05 c c of packed sheep cells were added to 1 c c of hemolysin (serum A), the corpuscles went to the bottom of the tube in a few seconds. The effect of these hemagglutinins was to prevent the corpuscles from being in continuous contact with the hemolysins even on frequent shaking. The lack of constancy in results, therefore, is what one might have expected.

TABLE 5

THE EFFECT OF THE CONCENTRATION OF HEMOLYSIN ON THE ABSORPTION CAPACITY OF 0.05 C C OF PACKED SHEEP CORPUSCLES AFTER EXTRACTION FOR 10 MINUTES AT ROOM TEMPERATURE

Hemolysin A				Hemolysin B			
Tubes	Number of Hemolysin Units per C c	Number of Units Remaining after Extraction	Number of Units Etxracted	Tubes	Number of Hemolysin Units per C c	Number of Units Remaining after Extraction	Number of Units Extracted
1	166	0	166	1	212	0	212
2	333	0	333	2	425	3	422
3	666	10	656	3	850	11	839
4	1,332	33	1,299	4	1,700	33	1,667
5	1,998	75	1,923	5	2,550	109	2,441
6	2,664	125	2,539	6	3,400	200	3,200
7	3,330	200	3,130	7	4,250	266	3,984
8	6,660	830	5,830	8	8,500	1,250	7,250
9	9,990	2,000	7,990	9	17,000	5,000	12,000
10	13,320	5,000	8,320	10	25,500	10,000	15,500
11	19,980	9,180	10,800	11	34,000	20,000	14,000
12	26,640	16,000	10,640	12	38,250	20,000	18,250
				13	42,500	25,000	17,500

It will be noted also that in view of the large range of hemolysin units employed, logarithmic paper was used in making these curves.

A consideration of the laws which govern the absorption of anti-sheep hemolysin by sheep cells is reserved for further studies. This, however, is established, namely, that 0.05 c c of packed sheep corpuscles are capable of absorbing as many as 18,000 units of hemolysin after 10 minutes' extraction at room temperature.

3. THE TIME AND TEMPERATURE OF SENSITIZING RED CELLS IN THE PRESENCE OF HEMOLYSIN

The foregoing studies suggested still another phase in connection with the union of sheep cells and hemolysin. We have in mind the

element of dissociation. Thus, it was observed that when 0.05 c c quantities of packed sheep cells were added to 1 c c solutions of hemolysin, each containing 400 units, more units were absorbed in some cases after 10 minutes' extraction at room temperature than after one hour extraction at water bath temperature. More frequently, the number of units absorbed after 10 minutes' extraction at room temperature was equal to the number of units absorbed after 1 hour in the water bath. The results of two such experiments are given in table 6.

There are indeed two possible factors which are liable to interfere with the absorption of hemolysin by sheep cells after an exposure of 1 hour in the water bath: first, the probable element of dissociation

TABLE 6

THE EFFECT OF TIME AND TEMPERATURE ON THE HEMOLYSIN ABSORPTION CAPACITY OF 0.05 C C PACKED SHEEP CELLS

Hemolysin C					Hemolysin D				
Tubes	Number of Units per C c	Temperature of Extraction	Time of Extraction, Min.	Units Extracted	Tubes	Number of Units per C c	Temperature of Extraction	Time of Extraction, Min.	Units Extracted
1	9,990	Water bath	10	6,660	1	7,500	Water bath	10	5,190
2	9,990	Room	10	6,240	2	7,500	Room	10	5,190
3	9,990	Water bath	60	6,240	3	7,500	Water bath	60	4,800
4	9,990	Water bath	120	6,240	4	7,500	Water bath	120	4,420

during this period; second, the possibility of hemolysis of a small number of corpuscles, liberating thereby a small quantity of hemolysin. A trace of hemolysis was indeed observed in hemolysin 2 (table 3) after centrifuging the tubes which were kept in the water bath 1 hour and longer.

SUMMARY

A quantitative study of some factors which govern the affinity of sheep corpuscles for antisheep hemolysin was carried out. The hemolysin was obtained by immunizing rabbits with sheep corpuscles in the usual manner. The corpuscles employed were obtained interchangeably from 5 different sheep. A unit of hemolysin was taken to be the smallest quantity which completely hemolyzed 0.1 c c of a 5% suspension of sheep cells in the presence of 0.1 c c of pooled guinea-pig complement after 15 minutes' incubation in the water bath.

In the first series of experiments, the rate of absorption of antisheep hemolysin by sheep corpuscles at different temperatures was

studied. The extraction periods were 5, 10, 15 and 20 minutes, and the temperatures were water bath, room and icebox. The hemolysin was extracted from both salt and pooled serum solutions. It was found, when 0.05 c c quantities of packed sheep corpuscles were added to 1 c c quantities of either salt or serum solutions, each containing 400 units of hemolysin, that the differences in the quantity of hemolysin absorbed at extraction periods of 5 to 20 minutes were not marked Neither were there large differences in the number of units absorbed at water bath and icebox temperatures (table 1).

In the second series, the effect of the concentration of hemolysin on the absorption capacity of 0.05 c c of packed sheep cells was studied. The extraction was in each case carried out for 10 minutes at room temperature. It was shown that the number of hemolysin units that this quantity of sheep corpuscles is capable of absorbing is directly proportional to the concentration of hemolysin. The largest number of units that 0.05 c c of packed cells absorbed in these experiments was 18,000. This number, however, does not represent the true absorption capacity of this quantity of sheep cells. The hemolysin serums employed, either undiluted or in low dilution, contained in every instance large numbers of hemagglutinins. These tended to bring about immediate precipitations of the corpuscles and thereby prevented proper contact between the hemolysin and the cells.

Finally, the effect of time and temperature on the hemolysin absorption capacity of 0.05 c c of packed sheep cells was studied. It was found that a 10 minute extraction period at room temperature was in most cases equivalent to a 1 hour or 2 hour extraction period at water bath temperature. In a few cases, there was less extraction after 1 hour or 2 hours in the water bath compared with 10 minutes' extraction at room temperature. This is believed to be due to dissociation of hemolysin and cells after prolonged extraction at 37.5 C. and also to the hemolysis of some corpuscles at this temperature with the liberation of some hemolysin.